



Real-Time PCR Diagnostics for Detecting and Identifying Potential Bioweapons

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USAMRIID - Ft. Detrick

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Overview

- DSD Overview
- Determine gene target(s)
- Design specific probes
- Design specific primers
- Optimize assay conditions
- Determine limits of detection
- Test for cross reactivity, interference
- Multiplexing



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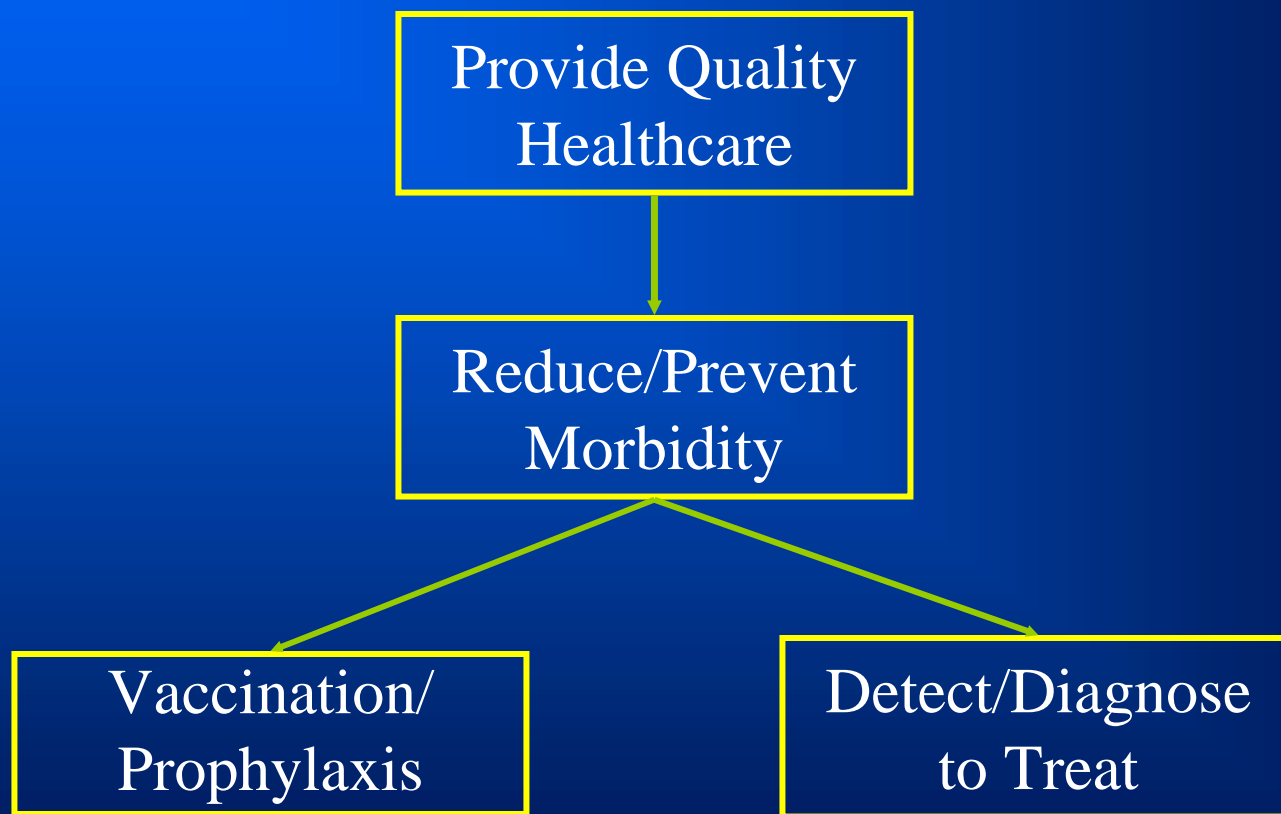
Diagnostics Systems Division

- Primary mission: research & development to advance diagnostic technologies for detecting biological agents
- Five branches
 - Applied Diagnostics (Immunoassay development)
 - Systems Development (Nucleic acid assay development)
 - Field Operations & Training (transition lab assays to field environments)
 - Clinical Pathology (USAMRIID clinical lab)
 - Special Pathogens Sample Testing Laboratory (SPSTL)



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Diagnostics and Patient Care: Goals



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Diagnostic Essentials

- Speed
- Accuracy
 - Sensitivity
 - Specificity



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Impact of Diagnostics on Patient Care

- Immediate postexposure (up to 24 h)

- ✓ very low concentration of agent

- ✓ **IMPACT**

- Acutely ill

- ✓ low concentration of agent

- ✓ **IMPACT**



- Critically ill

- ✓ High concentration

- ✓ **IMPACT**



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Diagnostic Time Constraint

- Usable results in under 24 hrs
- Issues
 - Detection of an event
 - Transport of samples
 - Diagnostic testing
 - Reporting results



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Classical Methods for Identifying Biological Agents

Method	Time	Sensitivity	Specificity
Culture Isolation	1 - 30 days	high	high
Animal Inoculation	2 - 30 days	high	high
Antigen Detection	4 - 18 hrs	low to high	high*
Antibody Detection	4 hr - 10 days	high	high*
Nucleic Acid Detection	3-8 hrs	high	high*

* reagent dependent



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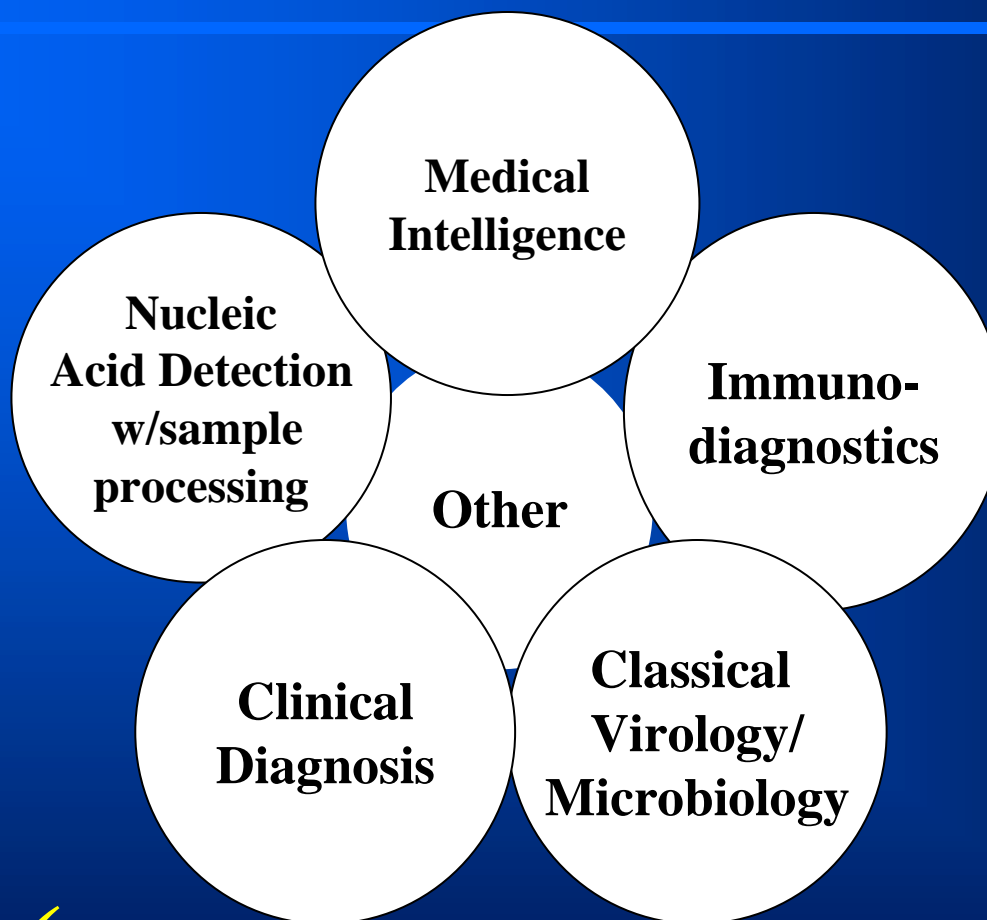
Diagnostic Sensitivity Requirements

Agent	Infective Dose	Agent	Infective Dose
Anthrax	8,000 to 50,000 spores	Smallpox	10-100 organisms
Brucellosis	10-100 organisms	VEE	10-100 organisms
Plague	100-500 organisms	Viral Hemorrhagic Fevers	1-10 organisms
Q-fever	1-10 organisms	Botulinum Toxins	~70 ng
Tularemia	10-50 organisms	Staph Enterotoxin B	~30 ng



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Integrated Identification and Diagnostic System



✓ *Definitive biological agent diagnosis
requires integrated identification technologies*



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Real-Time PCR

- Rapid

- < 30 minutes for DNA targets
- < 45 minutes for RNA targets

- Sensitive

- Potential for single copy gene detection

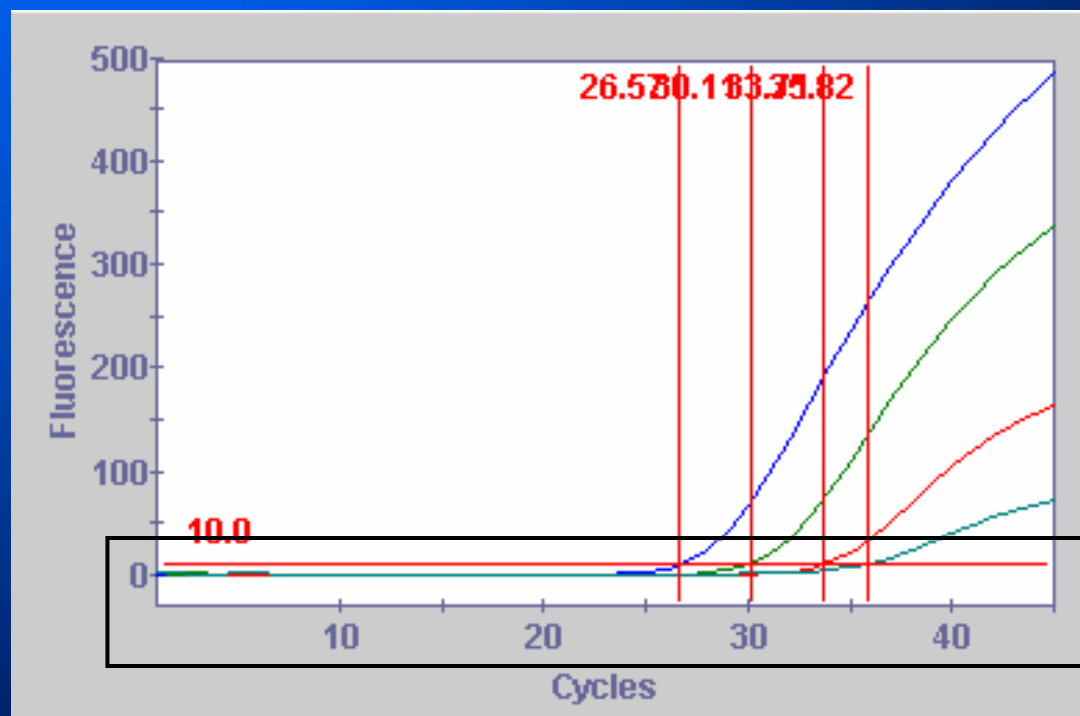
- Specific

- Added specificity of probe



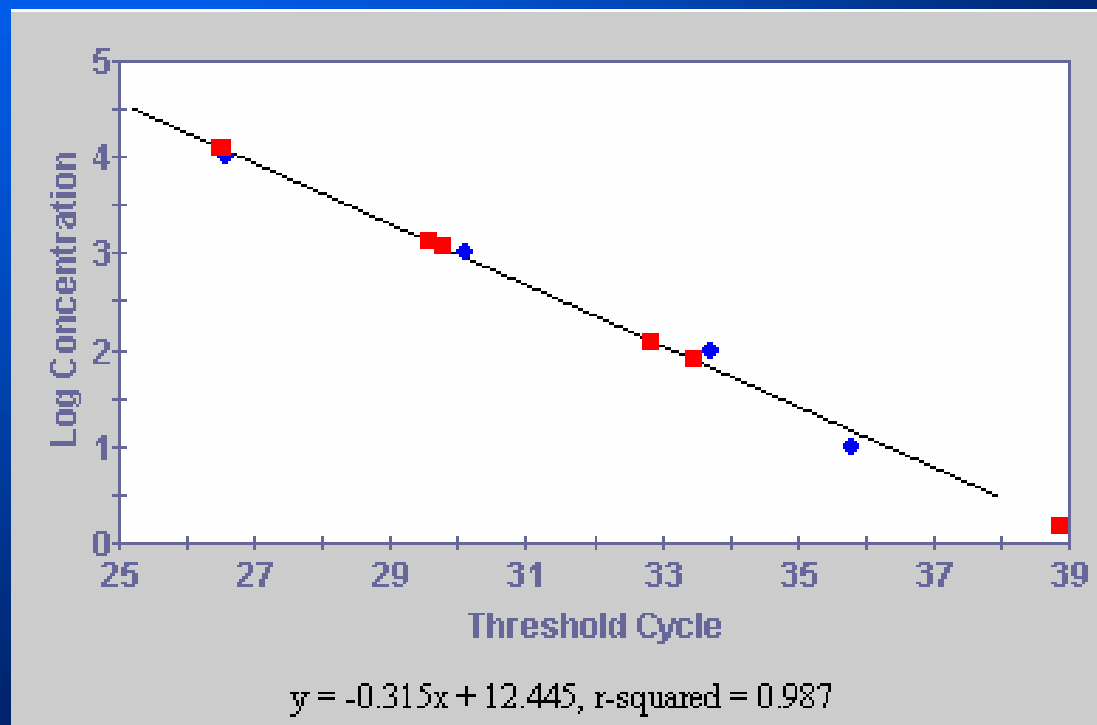
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Real Time PCR



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Quantitative PCR



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Sample Types

Medical specimens

- swabs
- whole blood and serum
- urine
- feces
- sputum
- lesion exudate
- tissues

Environmental samples

- air samplers/collectors
- swabs
- water
- soil

✓ Each matrix may require a unique processing protocol.



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Biomarkers

Diversity & Depth



Specific virulence markers

Genus and species markers

**Common pathogenic markers
& antibiotic resistance**

Host Response Markers



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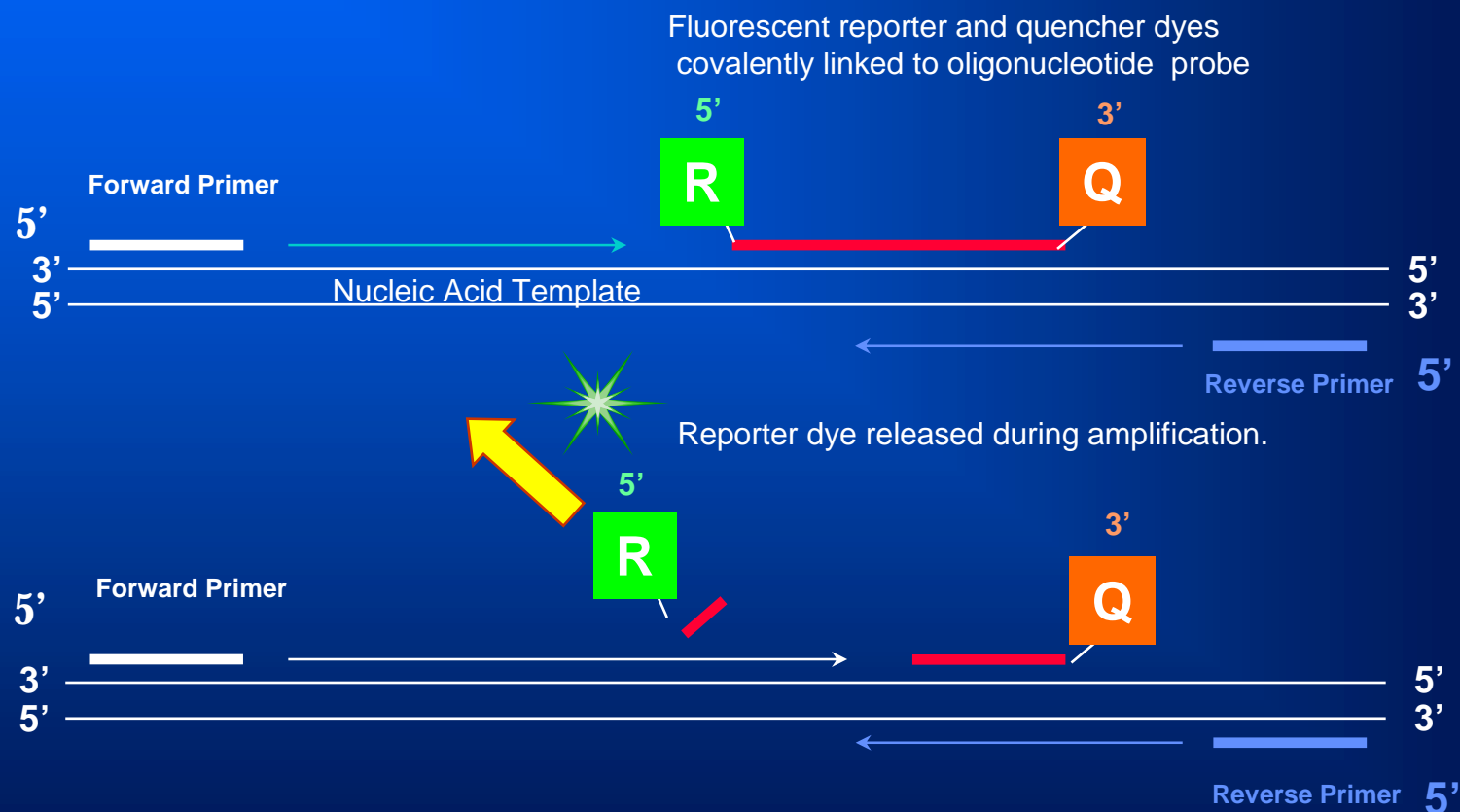
Determine Gene Target(s)

- Targets that are specific to agent of interest
 - Specific virulence (toxin genes)
 - Specific function (accessory genes)
- Genes that code for protein product
- Exploit sequence variability for allelic discrimination



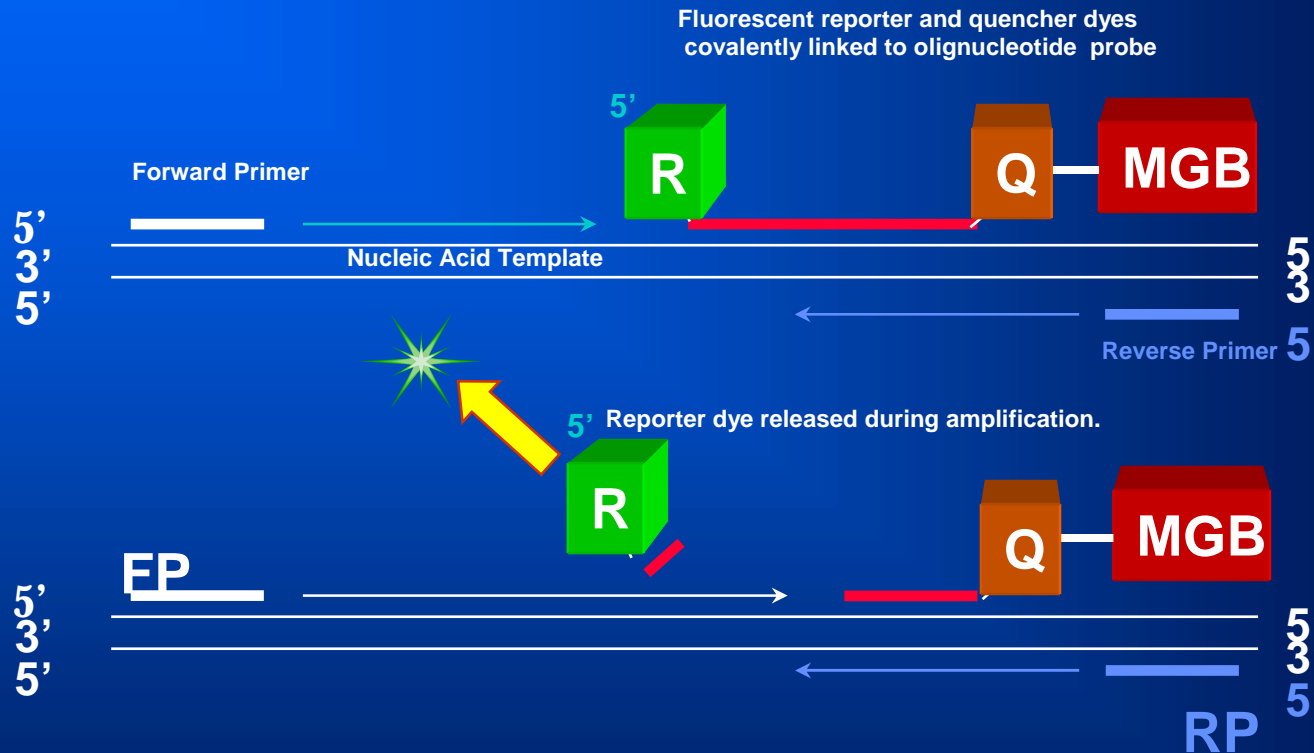
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Taqman[®] Chemistry



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Common Real-Time PCR Chemistry: TaqMan[®]-MGB



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Primer Design Guidelines

- Length of 18-25 nucleotides
- Primer Tms of 59⁰-60⁰ C, both primer Tms within 2⁰C
- No more than 2 Gs or Cs within the 5 terminal nucleotides at the 3' end
- The primers corresponding to the same strand as the probe should be within 30 nucleotides of the probe
- Avoid long runs of single nucleotides
- 40-60% GC content
- Amplicon of 80-150 base pairs



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Probe Design Guidelines

- Length of ≤ 30 oligonucleotides
- Probe T_m 7⁰-10⁰ C higher than primer T_m s (67-70⁰C)
- No 5' terminal G residues
- Less than 30 nucleotides from corresponding strand's primer
- Avoid long runs of single nucleotides
- Select strand which gives probe more C than G residues
- GC content 40-60%



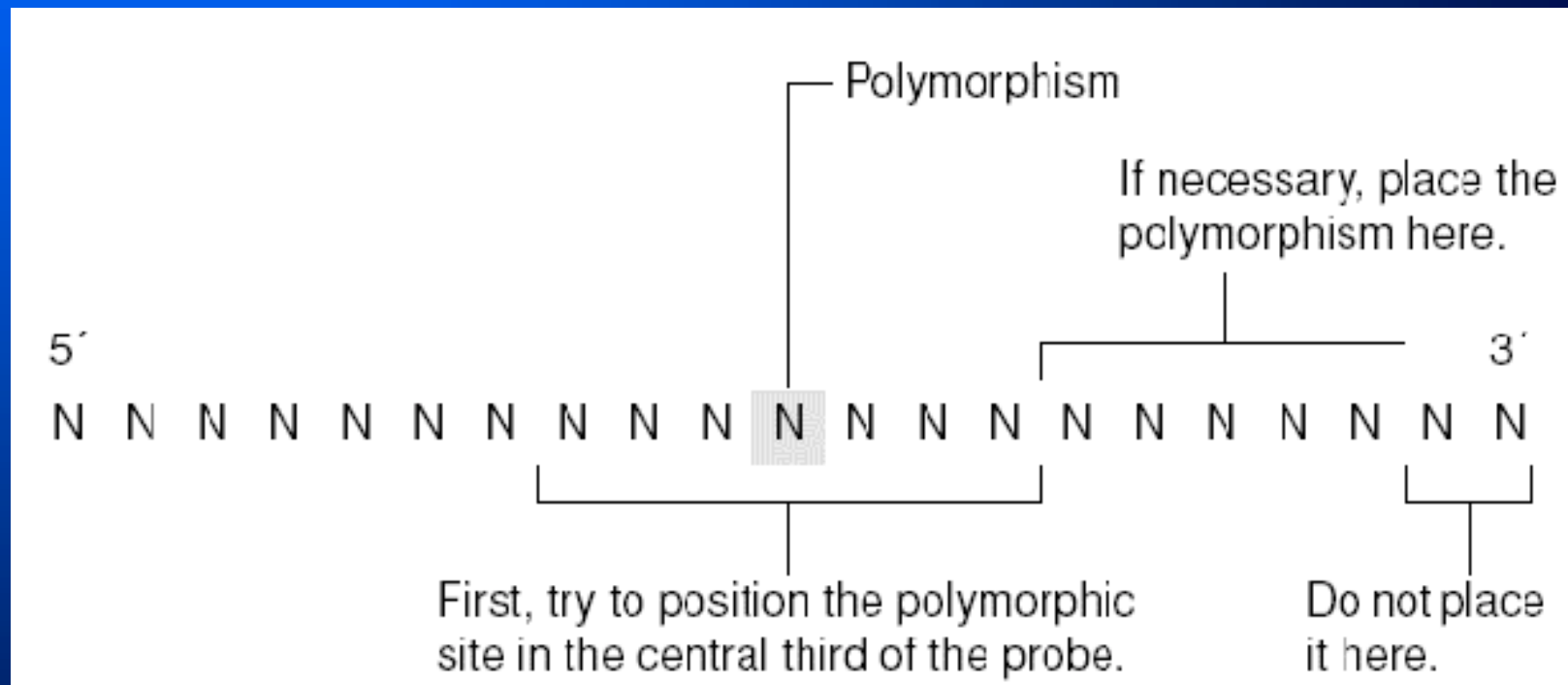
Taqman[®] MGB Probes

- Licensed to Applied Biosystems by Epoch biosciences
- Protein which binds double stranded DNA within the minor groove
 - Attached to 3' end of probe
 - Folds back and stabilizes the duplex after hybridization
 - Stabilization raises the T_m of the probe by 10-12°C
 - » Shortens probe length
 - » Improves allelic discrimination
 - » Provides greater flexibility in probe design
- Probes are coupled with a non-fluorescent quencher at the 3' end



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Taqman[®] MGB Probes for Allelic Discrimination



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Design Software

- Primer Express 2.0 – Applied Biosystems
- LightCycler Primer and Probe Design
- NetPrimer (Premier Biosoft)
- OMIGA 2.0
- Oligo



Taqman[®] Optimization

- Optimize the following parameters
 - MgCl_2 concentration (3mM-7mM)
 - Primer concentration (0.1 μM - 1.0 μM)
 - Temperature (Smart Cycler)
 - Probe
- Criteria for optimal conditions
 - Conditions which result in the earliest crossing threshold (Ct)
 - Conditions which produce the most fluorescence (endpoint fluorescence)
 - All conditions assayed in triplicate - average of triplicates are used for comparisons



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Chemistry

- Buffers from Idaho Technologies
 - dNTPs and 10X buffer with MgCl_2
- Smart Cycler™ Additive Reagent (SCAR buffer), Cepheid technical note
- Platinum Taq DNA Polymerase (Invitrogen)



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Typical Cycling Parameters

- Two-step PCR

- Denature
- Extend and anneal

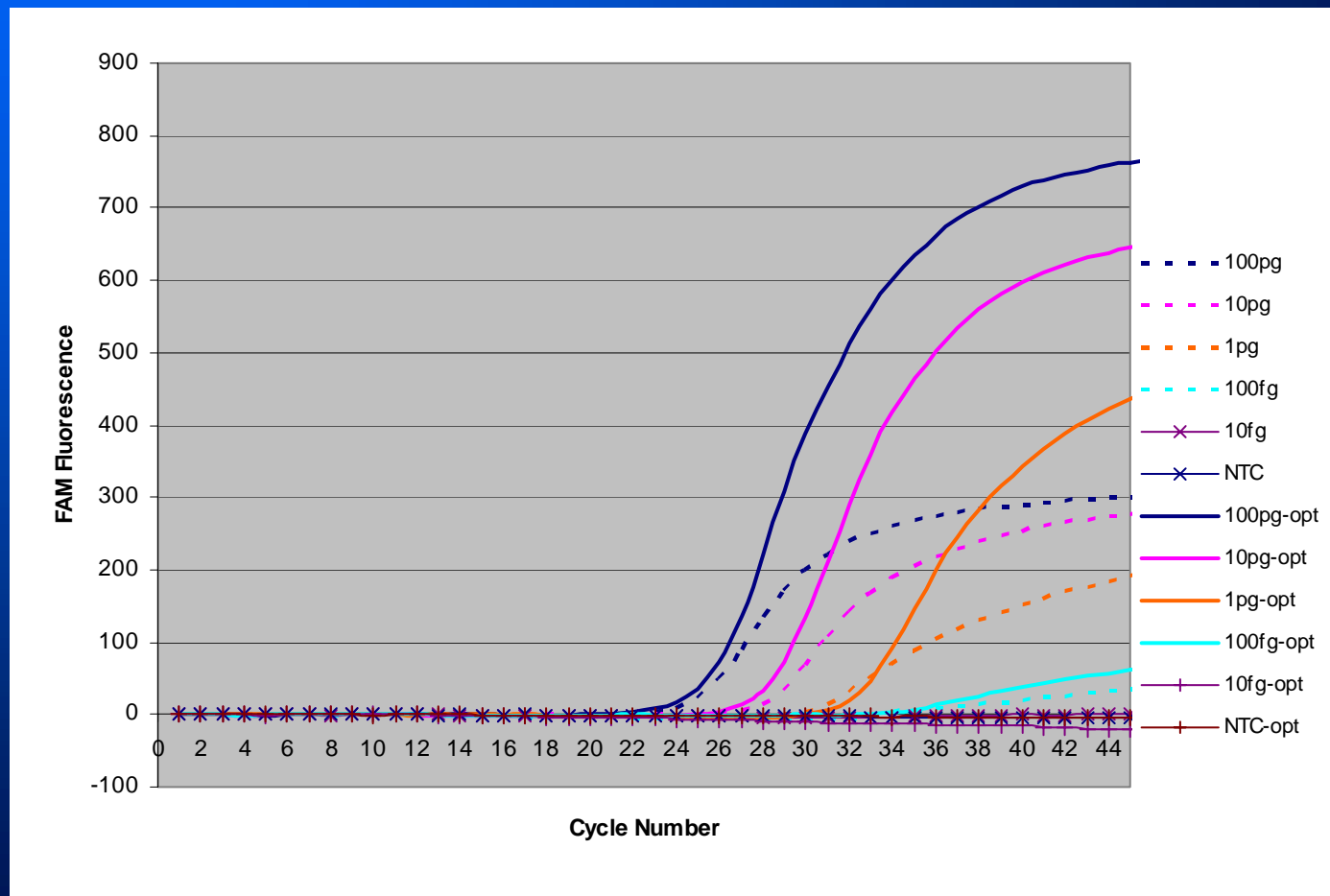
- Parameters

- 95°C for 2 minutes (activates Taq polymerase)
 - 95°C for 1 second
 - 60°C for 20 seconds
- 45 cycles

- Total time <30 minutes



Sensitivity / Optimized Assay



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Specificity

Organism

Acineobacter baumannii
Bacillus anthracis BA0068
Bacillus anthracis
Bacillus anthracis
Bacillus anthracis N.H.
Bacillus anthracis (Ames)
Bacillus anthracis (Sterne)
Bacillus anthracis (Buffalo)
Bacillus anthracis (ST1)
Bacillus anthracis (SPS.97.13.079)
Bacillus anthracis (SPS 97.13.213)
Bacillus anthracis (V770-NP-1R)
Bacillus anthracis (CDC 476)
Bacillus anthracis (Vollum)
Bacillus anthracis (NH)
Bacillus cereus
Bacillus cereus
Bacillus thuringiensis
Bacillus coagulans
Bacillus macerans
Bacillus megaterium
Bacillus popilliae

Organism

Bacillus subtilis var niger
Bacillus bronchiseptica
Clostridium botulinum
Comamonas acidivarns
Corynebacterium sp.
Enterococcus durans
Enterococcus faecalis
Enterococcus faecalis
Escherichia coli
H. influenzae
Klebsiella Pneumoniae
Neisseria lactamica
Proteus mirabilis
Providencia stuartii
Pseudomonas aeruginosa
Ralsonia picketti
Salmonella enteritidis
Serratia odorifera
Shigella flexneri
Shigella sonnei
Staphylococcus aureus
Staphylococcus hominis

Organism

Staphylococcus saprophyticus
Staphylococcus epidermidis
Staphylococcus Aureus
Streptococcus pyogenes
Streptococcus pneumoniae
Yersinia enterocolitica
Yersinia kristensenii
Yersinia frederiksenii
Yersinia pseudotuberculosis
Yersinia ruckeri
Yersinia enterocolitica
Yersinia pestis
Yersinia pestis
Yersinia pestis
Yersinia pestis
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Yersinia pestis
Yersinia pestis
Yersinia pestis
Yersinia pestis



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Interference

Ct values

Sample	no spike	5 ng Hu DNA spike
	AVE	AVE
100pg	24.05	24.20
10pg	28.20	27.76
1pg	31.62	32.31
100fg	36.32	37.11
10fg	0.00	0.00

Endpoint fluorescence

Sample	no spike	5 ng Hu DNA spike
	AVE	AVE
100pg	464.85	442.60
10pg	358.03	330.98
1pg	218.81	128.91
100fg	27.13	9.33
10fg	-1.67	-5.51



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Assays Available

- *Bacillus anthracis*
- *Yersinia pestis*
- *Brucella* sp.
- *Burkholderia mallei* / *pseudomallei*
- *Clostridium botulinum* toxins
- *Coxiella burnetti*
- *Francisella tularensis*
- *Orthopox* Species
- *Variola*
- Monkeypox
- *Staphylococcus aureus* toxins
- SARS
- Filoviruses



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